

Sustained Expression of Steroid Receptor Coactivator SRC-2/TIF-2 is Associated with Better Prognosis in Malignant Pleural Mesothelioma

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Introduction: Estrogen receptor beta (ER β) overexpression by malignant pleural mesothelioma (MPM) tumor cells correlates with enhanced patient survival. ER-regulated transcription is mediated by the p160 family of steroid receptor coactivators (SRCs), and SRC isoform overexpression is associated with worse prognosis in many steroid-related malignancies. The aim of this study was to establish whether SRC isoform expression varied between individual MPM tumors with positive or negative prognostic significance.

Methods: Immunohistochemical analysis of tumor biopsies from 89 subjects with confirmed histological diagnosis of MPM and biopsies from 3 normal control subjects was performed to detect the expression of SRC-1, SRC-2 (TIF-2), SRC-3 (AIB-1), and ER β . Allred scores for expression of ER β and each of the SRCs were determined, and Kaplan-Meier survival curves were calculated to correlate biomarker expression, gender, and histology type with postdiagnosis survival.

Results: ER β and all the SRCs were expressed at high levels in normal pleural mesothelium, and expression of each biomarker was reduced or lost in a subset of the MPM subjects; however, postdiagnosis survival only significantly correlated with TIF-2 expression. Low or intermediate expression of TIF-2 correlated with reduced median postdiagnosis survival (9 months) compared with those subjects whose tumors highly expressed TIF-2 (20 months) ($p = 0.036$, log-rank test).

Conclusions: Maintained high expression of TIF-2 in tumor cells is a positive prognostic indicator for postdiagnosis survival in patients with confirmed MPM. This is the first clinical study to correlate high TIF-2 expression with improved patient prognosis in any malignancy.

Key Words: Mesothelioma, Steroid receptor coactivator, Estrogen receptor.

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Malignant pleural mesothelioma (MPM) is a rare but highly aggressive tumor that arises from the mesothelial surfaces of the pleura.¹ A causative link has been established between exposure to asbestos fibers and the subsequent development of MPM in 85% of subjects, while simultaneous infection with SV40 virus is also believed to contribute to MPM etiology.² MPM is often detected decades after initial exposure to the carcinogen; however, there is also a genetic contribution that predisposes individuals to developing this malignancy.³ MPM is invariably terminal, and histological subtyping is currently the most important prognostic indicator of postdiagnosis survival time.⁴ Patients diagnosed with epithelioid MPM have a mean postdiagnosis survival period of 16.3 months, while patients with sarcomatoid or biphasic MPM have a poorer prognosis with mean postdiagnosis survival times of 6.1 and 9.5 months, respectively.⁵ The estimation of prognosis based solely on histological typing is imprecise as up to 70% of all MPM cases are of epithelioid type, and considerable variation occurs in the actual survival times for patients within this subgroup. Additional prognostic markers are required to further subtype MPM cases and so more effectively refine patient treatment regimes.

Female gender is a positive prognostic factor in MPM survival,^{5–7} and this protection is at least partially mediated through the action of estrogen receptor beta (ER β).⁸ ER-mediated transcription initiation occurs when ligand-bound ER interacts with gene promoter sequences and recruits steroid receptor coactivators (SRCs) to promote the formation of the transcription preinitiation complex.⁹ The p160 family of coactivator proteins make a rate-limiting contribution to the formation of this complex and so rigidly control transcriptional responses stimulated by all steroid hormones. The p160 family is comprised of steroid receptor coactivator-1 (SRC-1/NCoA-1),¹⁰ transcriptional intermediary factor 2 (TIF-2/SRC-2/GRIP-1/NCoA-2),¹¹ and amplified in breast cancer-1 (AIB-1/SRC-3/NCoA-3).¹² Dysregulation of p160 SRC expression affects tumor cell proliferation and

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invasive capacity; this occurs in all steroid-sensitive malignancies including prostate carcinoma¹³ and endometrial carcinoma.¹² The contribution of SRCs to tumorigenesis is best characterized in the context of breast carcinoma where AIB-1 is defined by its upregulation in the most aggressive tumors¹² and confirmed by the observation that AIB-1 overexpression in a mouse model resulted in the spontaneous generation of mammary tumors.¹⁴

The overall effect of estrogen action on tumor cell biology is complex, particularly in tissues that express both ER α and ER β .¹⁵ There is growing evidence of a protective role for ER β expression and activation in some malignancies, including colorectal carcinoma¹⁶ and MPM.⁸ In the context of colorectal carcinoma, ER β is highly expressed by differentiated cells of the colonic epithelium and its expression is lost in the most aggressive of tumors and correlates with poor prognosis.¹⁶ The dependency of this ER β tumor suppressor effect on differential SRC expression has not been investigated and raises the possibility that in specific, ER β -attenuated malignancies, SRCs may also have a tumor-suppressive role. Indeed, in colon carcinoma, the elevated expression of AIB-1 correlates with improved patient survival.¹⁷ Thus, in contrast with estrogen-stimulated malignancies such as breast carcinoma or endometrial carcinoma, the loss of specific SRC expression could diminish the protective effects exerted by ER β and so be a negative prognostic indicator. The aims of this study were to evaluate expression of SRC-1, TIF-2, and AIB-1 in tumors from a cohort of patients with confirmed MPM diagnosis using immunohistochemistry (IHC), to relate SRC expression in tumor cells to patient survival, and to compare the prognostic efficacy of SRC isoform expression with other indicative parameters of postdiagnosis survival.

PATIENTS AND METHODS

Patients and Tissue Samples

Eighty-nine confirmed cases of MPM and three control subjects were identified from the archival pathology files of the Pathology Unit of the Regional Hospital of Mestre-Venice, Italy. All diagnoses of MPM were based on World Health Organization criteria and confirmed in all instances by clinical, morphological, and IHC data. The tissue samples were taken after videothoracoscopy biopsy or from resected surgical specimens. The tissue samples were fixed in neutral formalin and embedded in paraffin. Permission for tissue to be used for research purposes was obtained according to local ethical procedures and following informed patient consent. Clinical data relating to each of the subjects were obtained with consent from primary patient records and coded before analysis by researchers.

Immunohistochemistry

IHC parameters for the ER β and p160 SRC family specific-antibodies were initially optimized using a breast carcinoma tissue microarray. IHC analysis for each antigen was performed using a Bond III Automated IHC Stainer (Leica Microsystems, Wetzlar, Germany) on serial 4- μ m depth tissue sections from each of the embedded specimens.

Slides were treated for 20 minutes with Leica BondMax Epitope Retrieval Solution 1 (ER1) for detection of TIF-2 and Leica BondMax Epitope Retrieval Solution 2 (ER2) for detection of SRC-1, AIB-1, and ER β to achieve postsectioning antigen retrieval. Specific primary antibodies were applied as indicated: SRC-1 (Clone 128E7, rabbit monoclonal antibody, 1:200; Cell Signaling Technology, Danvers, MA), TIF-2 (Clone 29, mouse monoclonal antibody, 1:100; BD Transduction Laboratories, San Jose, CA), AIB-1 (Clone 34, mouse monoclonal antibody, 1:200; BD Transduction Laboratories, San Jose, CA), and ER β (NCL-ER β , mouse monoclonal antibody, 1:100; Novocastra, Leica Microsystems, Ashbourne, Ireland). Primary antibodies were revealed using the Leica Bond Polymer Refine detection kit, and the signal was enhanced using the Leica BondMax DAB enhancer kit. Slides were counterstained with hematoxylin before mounting and microscopic visualization.

Scoring System

Semiquantitative determination of SRC-1, TIF-2, AIB-1, and ER β was performed according to the criteria described by Allred et al.¹⁸ The proportion of positive stained cells was rated as 0 = no cells stained positive, 1 = between 0% and 1% positive, 2 = between 1% and 10%, 3 = between 10% and 33%, 4 = between 33% and 66%, and 5 = between 66% and 100%. In addition to the proportion score, an intensity score was made on the basis of the average intensity of staining: 0 = negative, 1 = weak, 2 = intermediate, and 3 = strong. The proportion score and the intensity score were added to obtain the total score and is either 0 or between 2 and 8. Allred scores of 6 or greater were interpreted as strong staining. Slides were independently evaluated and scored in a blind fashion by two independent observers. Any discrepancies in scoring between the observers were resolved by review of the slides under a double-headed microscope, and a consensus score was allocated.

Statistical Analysis

Differences in postdiagnosis survival were calculated using Kaplan-Meier analysis, and survival curves were generated using the TMA Foresight package (TMA Foresight 3.01, Premier Biosoft International, Palo Alto, CA). *p* values were calculated using a log-rank test,¹⁹ and a *p* value less than 0.05 was taken to be indicative of statistical significance between populations. The multivariate Cox proportional hazard regression model was then used to calculate hazard ratios and to stratify the variables for known clinical parameters.²⁰ This ensures that the prognostic markers are independent of known clinical parameters (gender, tumor histological type, age at diagnosis, and chemotherapeutic intervention). All calculations were performed using the survival library of the open source R package (<http://cran.r-project.org/>), and the two-tailed Wald test was applied.

RESULTS

Patient Cohort Characteristics

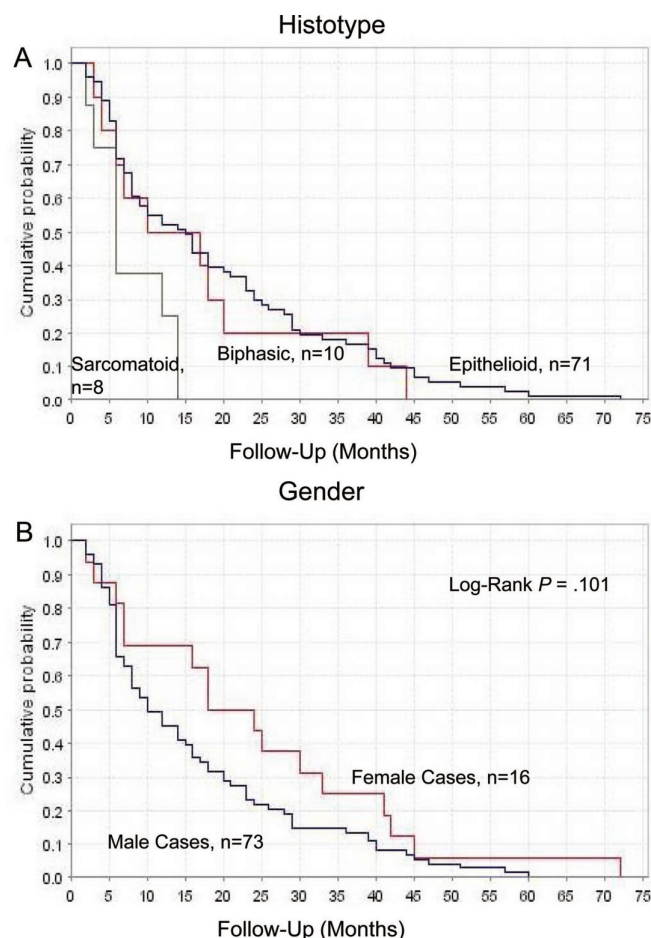
SRC isoform and ER β expression were determined using IHC in tumor biopsy specimens for a well-defined

TABLE 1. Summary of Subject Characteristics at Diagnosis and p160 SRC Immunohistochemistry Data ($n = 89$)

	Male ($n = 73$)	Female ($n = 16$)	All ($n = 89$)
Age at diagnosis, median (range)	64 (44–82)	65 (55–79)	64 (44–82)
Histology type, n (%)			
Epithelioid	56 (76.7)	15 (93.7)	71 (79.8)
Biphasic	9 (12.3)	1 (6.3)	10 (11.2)
Sarcomatoid	8 (11.0)	0 (0)	8 (9.0)
Stage			
T2	22 (30.1)	5 (31.2)	27 (30.3)
T3	40 (54.8)	5 (31.2)	45 (50.6)
T4	11 (15.1)	6 (37.5)	17 (19.1)
N0	45 (61.6)	14 (87.5)	59 (66.3)
N1	16 (21.9)	0 (0)	16 (20)
N2	12 (16.4)	2 (12.5)	14 (15.7)
M0	73 (100)	16 (100)	89 (100)
M1	0 (0)	0 (0)	0 (0)
Surgery			
Yes	48 (65.75)	11 (68.75)	59 (66.3)
No	25 (34.25)	5 (31.25)	30 (33.7)

SRC, steroid receptor coactivator.

cohort of MPM subjects with more than 5 years postdiagnosis follow-up. Paraffin-embedded tumor tissue samples from 89 patients with age range 44 to 82 years (clinicopathological characteristics shown in Table 1) and 3 normal control subjects were analyzed. The gender distribution of the MPM cohort was 73 male and 16 female subjects; the median age at diagnosis was 64 years for male subjects and 65 years for female subjects. Histological types were determined to be 71 (79.8%) epithelioid, 10 (11.2%) biphasic, and 8 (9.0%) sarcomatoid. The majority of the subjects ($n = 64$) had received cisplatin-based chemotherapy postdiagnosis. Overall, postdiagnosis Kaplan-Meier survival curves for histological type and gender are shown (Figure 1). The median postdiagnosis survival time was 15 months for patients with epithelioid MPM, 10 months for subjects with biphasic mesothelioma, and 6 months for subjects with sarcomatoid mesothelioma (Figure 1A). The median survival time for male subjects was 10 months and 18 months for female subjects (Figure 1B). These survival data show that the MPM subjects in this study have equivalent distribution and outcomes when compared with MPM subject cohorts from other studies in which patients with the epithelioid-type mesothelioma have better prognosis, followed by patients with biphasic mesothelioma, while patients with sarcomatoid mesothelioma have the worst prognosis.^{5,7} Previous studies have also found a gender dichotomy in patient outcome, with improved survival for female MPM patients.^{5,7,8} Although not statistically significant for this cohort ($p = 0.101$, log-rank test), these results in common with other published studies also indicate that female MPM subjects have a better prognosis than their male counterparts.

**FIGURE 1.** Kaplan-Meier survival curves for malignant pleural mesothelioma subject survival discriminated by histological type (A) and gender (B).

Normal Pleura and MPM Tissue Tumor Samples Express SRC-1, TIF-2, and AIB-1

The p160 SRCs are required by all steroid nuclear receptors including the estrogen receptors to initiate ligand-dependent transcription.⁹ The upregulation of SRCs particularly AIB-1 in the context of breast carcinoma correlates with a more aggressive tumor phenotype in both estrogen-dependent and estrogen-independent tumors.¹⁴ ER β has been proposed as a tumor suppressor in colorectal carcinoma,²¹ and its overexpression in MPM correlates with enhanced patient survival.⁸ Comparative SRC expression in a MPM patient cohort has not been previously investigated. Herein, we found predominantly nuclear staining for each of the p160 SRCs: SRC-1 and AIB-1 (Supplemental Figure 1, <http://links.lww.com/JTO/A146>), TIF-2, and also ER β (Figure 2) in all three normal pleural mesothelium tissue samples. All cells making up the normal mesothelial monolayer stained with high intensity for ER β and each of the SRCs. For the 89 MPM subjects, there was marked variation in IHC staining intensity and the proportion of positively stained cells between the tumor specimens from the different subjects (Figure 2). The Allred scoring system was applied to quantify staining in each of the tumor samples for the

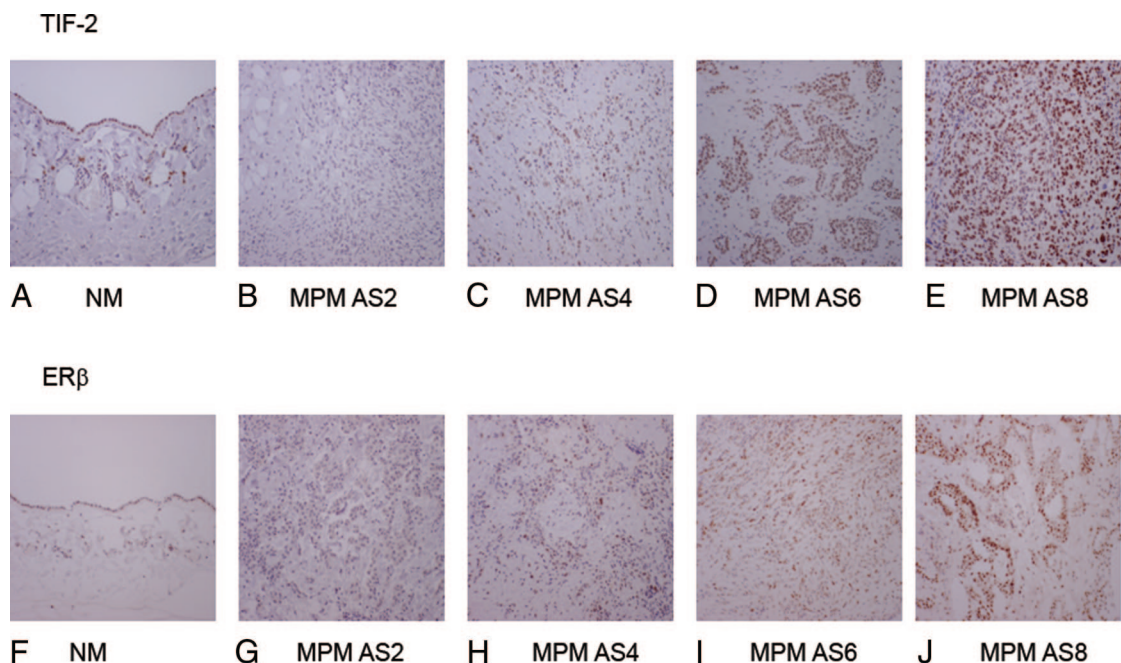


FIGURE 2. Representative immunohistochemical staining ($\times 200$ magnification) TIF-2 (A–E) and ER β (F–J) in normal pleural mesothelium (NM) and malignant pleural mesothelioma tumor tissue with Allred score (AS) values of 2, 4, 6, and 8.

TABLE 2. Summary of Subject p160 SRC and ER β Immunohistochemistry Data ($n = 89$)

	Male ($n = 73$)	Female ($n = 16$)	All ($n = 89$)
SRC-1 expression			
Low/intermediate	42 (57.5%)	8 (50%)	50 (56.2%)
High	31 (42.5%)	8 (50%)	39 (43.8%)
TIF-2 expression			
Low/intermediate	44 (60.3%)	7 (43.8%)	51 (57.3%)
High	29 (39.7%)	9 (56.2%)	38 (42.7%)
AIB-1 expression			
Low/intermediate	31 (42.5%)	6 (37.5%)	37 (41.6%)
High	42 (57.5%)	10 (62.5%)	52 (58.4%)
ER β expression			
Negative	19 (26%)	4 (25%)	23 (25.8%)
Positive	54 (74%)	12 (75%)	66 (74.2%)

SRC, steroid receptor coactivator.

three p160 SRCs and ER β (Table 2). A predominantly nuclear staining with Allred score ≥ 6 was observed in 39 (43.8%) MPM subjects for SRC-1, 38 (42.7%) subjects for TIF-2, and 52 (58.4%) subjects for AIB-1. Low or intermediate staining (Allred score ≤ 5) was observed in 50 cases for SRC-1 (56.2%), 51 cases for TIF-2 (57.3%), and 37 cases for AIB-1 (41.6%). ER β was also localized to the nuclei of positively stained cells, and negative ER β staining (Allred score ≤ 3) was observed in 23 cases (25.8%).

Kaplan-Meier survival curves were calculated for subjects with high versus intermediate/low Allred expression scores of SRC-1 (Supplemental Figure 2, <http://links.lww.com/JTO/A147>), TIF-2 (Figure 3A), AIB-1 (Supplementary data), and positive

versus negative ER β expression (Figure 4) in the individual tumor samples. Median postdiagnosis survival for subjects with high expression of SRC-1 was 14 months, while the median survival for subjects with intermediate/low expression was only slightly lower at 10 months. However, median survival for patients with high expression of TIF-2 was 20 months, while the median survival for patients with intermediate/low expression was significantly lower at 9 months, thus patients with intermediate/low expression of TIF-2 showed a statistically worse outcome compared with patients with high levels of TIF-2 expression ($p = 0.036$, log-rank test). Median survival for patients with high or intermediate/low expression of AIB-1 was 12 months. Median survival of ER β positive patients was 15 months and 8 months for ER β negative patients, but was not statistically significant in this cohort ($p = 0.254$, log-rank test).

TIF-2 Expression and Prognosis in Epithelioid and Male-Only Subgroups

Histological type and gender influence postdiagnosis survival in MPM. Epithelioid histological type is a positive prognostic factor in MPM, while male gender is a negative prognostic factor. To determine whether TIF-2 expression was of prognostic value when the outcome for epithelioid histology type or male subjects was analyzed separately, Kaplan-Meier survival curves were calculated for high versus intermediate/low TIF-2 expression in the tumors of subjects within these two subgroups. Median survival for subjects with epithelioid tumors ($n = 71$) that expressed high levels of TIF-2 was 23 months, while for those subjects with low or intermediate levels of TIF-2 expression had a median survival time of 9 months ($p = 0.031$, log-rank test) (Figure 3B). Median survival of male patients ($n = 73$) with high TIF-2

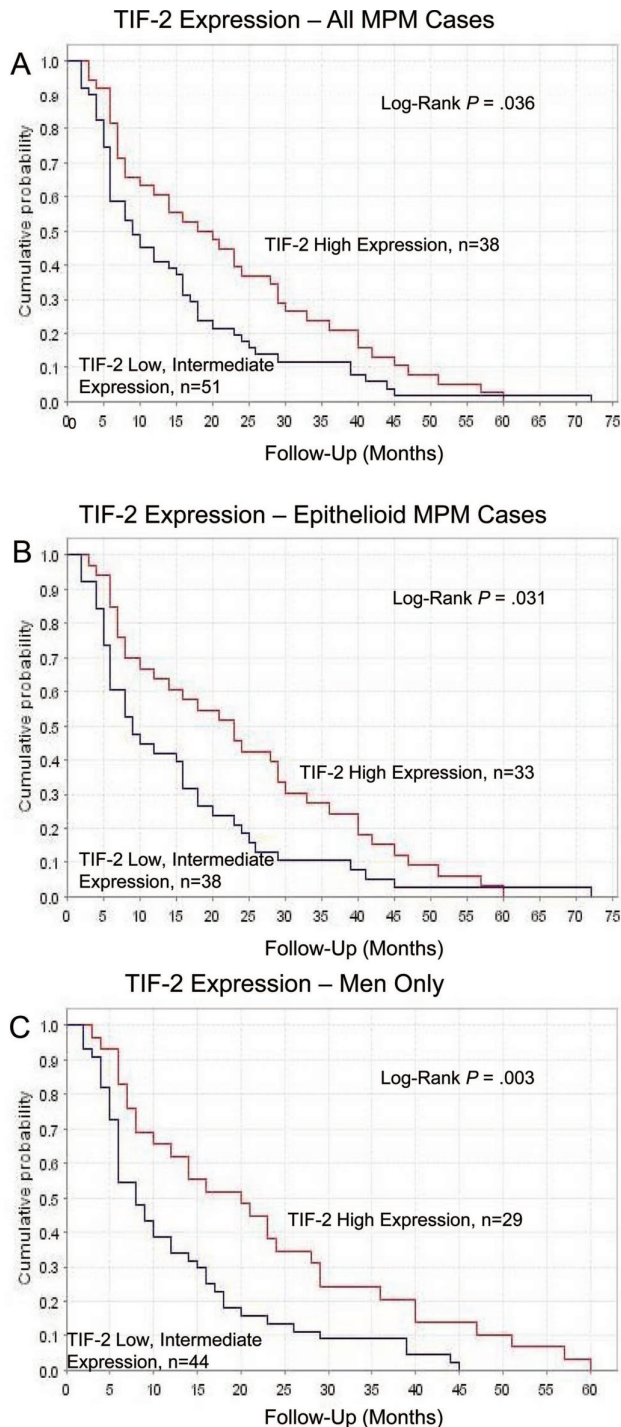


FIGURE 3. Kaplan-Meier survival curves for malignant pleural mesothelioma subject survival discriminated by high versus low/intermediate TIF-2 expression for the whole subject cohort (A), epithelioid cases only (B), and male subjects only (C).

expression levels was 20 months compared with 8 months for male patients with low or intermediate levels of TIF-2 expression ($p = 0.003$, log-rank test) (Figure 3C).

ER β Expression – All MPM Cases

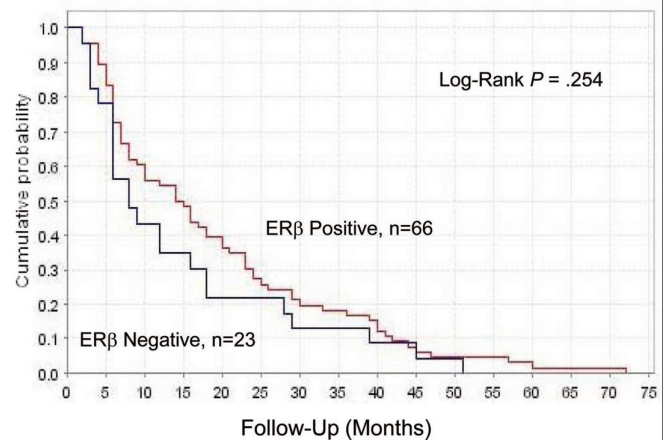


FIGURE 4. Kaplan-Meier survival curve for malignant pleural mesothelioma subject survival discriminated by positive versus negative/ER β expression for the whole subject cohort.

TABLE 3. Cox Proportional Hazards Model of Overall Survival ($n = 89$)

TIF-2 Expression	N	HR (95% CI)	p^a
Low/intermediate	51	1.00	
High expression	38	0.526 (0.301–0.918)	0.0239

^a Two-sided Wald test.

HR, hazard ratio; CI, confidence interval.

Hazard Ratio

Multivariate Cox proportional hazard regression analysis was performed correcting for gender (male, female); age at diagnosis (<60, 60–69, ≥ 70 years); histological type (epithelioid, biphasic, sarcomatoid) and chemotherapeutic intervention (therapy, no therapy) (Table 3). The adjusted hazard ratio for high versus low/intermediate TIF-2 expression was 0.526 (0.301–0.918) ($p = 0.0239$, two-sided Wald test).

DISCUSSION

MPM is a slow-developing malignancy with extremely poor patient outcome and limited availability of molecular markers to predict survival or therapeutic efficacy. In steroid-dependent malignancies of the reproductive tissues, overexpression of p160 SRC family proteins correlates with a more aggressive tumor phenotype and worse prognosis.¹⁴ The p160 SRCs form the scaffold for the assembly of the transcription preinitiation complex and as such are key components in the induction of estrogen-responsive genes. Dysregulation of p160 SRC action in breast carcinoma through overexpression and hyperphosphorylation of the coactivators results initially in amplification of estrogen responses in tumor cells and subsequently to uncoupling of cell growth from circulatory estrogen availability, contributing to the switch from endocrine-dependent to growth factor-dependent tumor cell proliferation.²² Recent data suggest that unusually MPM cell

proliferation may be slowed by circulating estrogens rather than accelerated,⁸ and consequently the molecular machinery of ER-regulated transcription is recruited to tumor suppression rather than promotion. The antitumor action of estrogens is transduced through ER β which regulates the expression of cell cycle regulators such as cyclin D1.^{8,23}

The strong dependency of ER transcriptional activity on SRC abundance led to our investigation of p160 SRC isoform expression in MPM tumors. Our study found that each of the p160 SRCs investigated, SRC1, TIF-2, and AIB-1, were highly expressed in normal pleural mesothelium from malignancy-free subjects, and this novel observation suggested a role for these cofactors in the normal physiological processes of the pleural mesothelium. Each of the SRCs were also expressed in the majority of the MPM tumor specimens analyzed; however, Allred immunohistochemical scoring of SRC expression revealed that there was a declining trend in the expression of all three SRCs in tumors from MPM subjects with the shortest postdiagnosis survival time. The decline in TIF-2 expression correlated most strongly with reduced postdiagnosis survival, and TIF-2 expression was a more accurate predictor of outcome than previously identified indicators of survival (histological type and gender) in this cohort of MPM subjects. Furthermore, TIF-2 was a better predictor of postdiagnosis survival within the epithelioid tumor subjects than in the cohort as a whole, which included the biphasic and sarcomatoid tumor subjects. The loss of TIF-2 expression thus identified a subgroup of epithelioid tumors that were most likely to have reduced postdiagnosis survival. Whether this discriminates those subjects whose malignancy is most likely to progress and develop a sarcomatoid tumor phenotype remains to be established.

ER β is implicated as a tumor suppressor not only in MPM but also in breast,^{24,25} prostate,²⁶ and colon carcinoma; however, TIF-2 expression correlates with worse prognosis in colorectal carcinoma because colonic epithelium also expresses ER α that requires the SRCs for its transcriptional activity.¹⁷ In general, ER α transduces the proliferative action of estrogen, particularly in reproductive tissues. As the normal pleural mesothelium expresses only ER β ,⁸ it may be anticipated that the SRCs participate exclusively in the anti-tumor actions of ER β promoted by circulatory estrogens. The benefits of ER β expression as manifest through slowed tumor cell growth may subside with the decline in TIF-2 expression that was observed in MPM subjects with the poorest postdiagnosis survival times. In this cohort of patients, ER β expression was a weaker predictor of survival than TIF-2 expression. This study is the first clinical report of the transcription factor TIF-2 acting in the role of a tumor suppressor and as a potential signaling intermediate underpinning the positive prognostic effects of ER β expression in MPM.

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